Virulence of *Xanthomonas oryzae* pv. *oryzae* on Rice Lines Containing Single Resistance Genes and Gene Combinations

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ABSTRACT

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Fifty isolates of *Xanthomonas oryzae* pv. *oryzae* were collected from different rice-producing districts of Nepal and evaluated for their virulence on these 11 rice lines having from one to four resistance genes: IRBB4 (*Xa4*), IRBB5 (*xa5*), IRBB7 (*Xa7*), IRBB8 (*xa8*), IRBB10 (*Xa10*), IRBB14 (*Xa14*), and IRBB21 (*Xa21*), two-gene combination AY4+5 (*Xa4* and *xa5*), three-gene combinations NH21-37-1-1 (*Xa4*, *xa5*, and *xa13*) and NH24-10-1-3 (*Xa4*, *xa5*, and *Xa21*), and four-gene combination NH56-1-44-4 (*Xa4*, *xa5*, *xa13*, and *Xa21*). The ability of an isolate to cause lesions with different lengths across the lines was interpreted as virulence. Isolates that were consistently associated with high or low virulence were statistically differentiated. Most isolates produced large lesions on near-isogenic lines with single genes and small lesions on lines with different gene combinations. Based on infection responses on the two-, three-, and four-gene combinations, five virulence groups were identified. Isolates in virulence group I were widely distributed. The line $\times$ isolate interactions were generally not significant with gene combinations, indicating a low possibility of specificity. A line with a four-gene combination, NH56, showed wider spectrum and higher level of resistance to *X. oryzae* pv. *oryzae* than the other lines. The results of this study will facilitate the deployment of effective resistance to *X. oryzae* pv. *oryzae* in Nepal.

Additional keywords: bacterial blight of rice, host resistance, *Oryza sativa*, pathogen variation

Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (26), is an economically important disease of rice in tropical Asia (17,18). Yield loss ranging up to 26% has been reported on susceptible rice cultivars (6). Host-plant resistance is an important component of an integrated management program for this disease. Twenty-one resistance genes (*Xa* genes) for BB have been identified by researchers worldwide (11,13,14,22), and some of these have been incorporated into modern rice varieties (13,28). However, compatible races of *X. oryzae* pv. *oryzae* have emerged, possibly as a result of selection pressure due to the presence of race-specific resistant cultivars (17,18,19,21). Surveys to characterize the virulence of *X. oryzae* pv. *oryzae* populations toward rice lines possessing known resistance genes

will provide important information that can be used in breeding programs to develop effective resistance in cultivars to be released in specific regions.

Both phenotypic and molecular markers have been used to analyze *X. oryzae* pv. *oryzae* populations in Nepal. Nine races of *X. oryzae* pv. *oryzae* have been characterized on the basis of interactions on five IRRI differentials and three Nepalese rice cultivars (5). Repetitive elements from *X. oryzae* pv. *oryzae* were used as probes in restriction fragment length polymorphism (RFLP) analysis and identified a high degree of genetic differentiation among the populations of this pathogen (7). Later, the genetic diversity of 171 isolates of *X. oryzae* pv. *oryzae* collected from 20 districts in eight rice-producing zones in Nepal was analyzed after polymerase chain reaction (PCR) using the rep-PCR ERIC primer and a primer (J3) based on an insertion sequence from *X. oryzae* pv. *oryzae*. Cluster analysis revealed that the 171 isolates formed five distinct clusters (2).

There are no satisfactory control measures for BB in Nepal because no immune rice genotypes have been detected among the rice germ plasms tested (4). Although the locally adapted cultivar Laxmi appears to possess partial resistance to BB (1; T. Adhikari et al., unpublished data), identi-

fying new sources of resistance is necessary to provide effective resistance because of the highly virulent isolates of *X. oryzae* pv. *oryzae* in Nepal. Recently, some single-, two-, three- and four-gene combinations of backcrossed rice lines containing varying assemblages of the BB resistance genes *Xa4*, *xa5*, *Xa7*, *xa8*, *Xa10*, *xa13*, *Xa14*, and *Xa21* have been developed at the International Rice Research Institute (IRRI) (11,14,22). These lines were developed by backcrossing resistance sources to the recurrent parent IR24, which is susceptible to all known Philippine races of the pathogen (22). The main objective of this study was to determine the relative virulence of *X. oryzae* pv. *oryzae* isolates from Nepal on rice lines containing single genes and gene combinations by phenotypic reactions.

MATERIALS AND METHODS

Bacterial isolates. Fifty isolates of *X. oryzae* pv. *oryzae* were collected during random disease surveys conducted in 11 rice-producing districts of Nepal between August and November 1996 (Table 1). Distances between the sites ranged from 1 to 350 km. At most of the sites, several rice fields showed symptoms of BB. A few infected leaf samples were collected at random from these fields. Bacteria were isolated from infected rice leaves as described by Adhikari et al. (5). A single colony was isolated and purified on peptone sucrose agar (PSA) medium (23) and maintained in 5% skim milk and PSA slants at 4°C for routine work. For long-term storage, the isolates were kept in 15% glycerol at -70°C. All the isolates collected were inoculated onto the susceptible rice cultivar IR24 to confirm their pathogenicity.

Rice lines. Seeds of the rice lines and IR24 were obtained from IRRI, Philippines, and used to characterize virulence of *X. oryzae* pv. *oryzae* isolates during the 1997 summer at the Institute of Agriculture and Animal Science (IAAS), Chitwan, Nepal. The seven near-isogenic lines with single genes *Xa4*, *xa5*, *Xa7*, *xa8*, *Xa10*, *Xa14*, and *Xa21*; a combination of *Xa4* and *xa5* genes NY4+5; the three-gene combinations NH21-37-1-1 (hereafter NH21) (*Xa4*, *xa5*, and *xa13*) and NH24-10-1-3 (NH24) (*Xa4*, *xa5*, and *Xa21*); and a four-gene combination NH56-1-44-4 (NH56) (*Xa4*, *xa5*, *xa13*, and *Xa21*) were tested in this study (Table 2). IR24 was used as a

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susceptible check. Rice seeds were first sown in a wooden seedbox in the greenhouse, and three similar seedlings of each line were transplanted to the 100-cm plastic buckets 10 days after sowing. Each plastic bucket was filled with a mixture of farm soil and farmyard manure at a ratio of 1:1. N-P-K was applied at the rate of 100-60-40 kg/ha (10-g mixtures of N-P-K per bucket) as the basal dose in the form of ammonium sulfate, superphosphate, and muriate of potash. Plants were irrigated daily and were top-dressed with urea at the rate of 60 kg/ha (5 g per bucket) 30 days after transplanting.

Inoculum preparation and inoculation. *X. oryzae* pv. *oryzae* isolates were transferred to PSA slants and incubated at 28°C for 72 h. A 3-day-old culture of each

isolate was used to prepare inoculum. Inoculum was prepared by suspending the bacterial cells in 10 ml of sterile distilled water and adjusting to a concentration of 10^8 CFU/ml prior to inoculation. The experimental unit consisted of three plants per treatment. Three fully expanded leaves of each plant in each bucket (total of nine leaves per experimental unit) were clip-inoculated with each isolate 40 days after sowing (12). The experiment was designed in a split plot with the rice line as main plot and the bacterial isolate as subplot (10). The entire experiment was performed twice.

Disease assessment. Lesion length from the cut leaf tip was measured in centimeters (cm) 14 days after inoculation (20). To characterize virulence, resistance or sus-

ceptibility was assessed from the nine inoculated leaves of each line per treatment. Disease reactions were categorized according to lesion length, where 0 to 3 cm was classified as resistant (R) and more than 3 cm as susceptible (S).

Statistical analyses. Lesion length data for each line-isolate combination were subjected to one-way analysis of variance (ANOVA). Means were separated by Fisher's least significance tests ($P < 0.05$). In addition, orthogonal contrasts were estimated to compare the cumulative effects of lesion lengths in the single genes and two-, three-, and four-gene combinations. All statistical analyses were performed using the SAS statistical package (Ver. 6.10, SAS Institute, Cary, NC). Virulence data of *X. oryzae* pv. *oryzae* isolates produced on the two-, three-, and four-gene combinations were used to generate the dendrogram using the Molecular Evolutionary Genetics Analysis (MEGA) program (15). Virulence was coded as "1" and avirulence as "0". Genetic distances were calculated based on pair-wise comparisons. To investigate the relationships between the isolates, cluster analysis was conducted from the genetic distances using the unweighted pair group method with arithmetic average mean (UPGMA).

RESULTS

All 50 isolates of *X. oryzae* pv. *oryzae* tested were virulent on rice lines containing the *Xa4*, *xa5*, *Xa7*, *xa8*, *Xa10*, *Xa14*, or *Xa21* resistance gene and on IR24, the susceptible check. Only a few isolates were virulent on the two-, three-, and four-gene combinations (Fig. 1). Based on the virulence or avirulence patterns on the two-, three-, and four-gene combinations, the 50 isolates of *X. oryzae* pv. *oryzae* were classified into five virulence groups (VG) using the UPGMA method of cluster analysis (Fig. 2). VG I consisted of 32 isolates that were virulent to the two-gene combination but avirulent to all three- and four-gene combinations (Table 3). VG II consisted of 10 isolates and were virulent to the gene combinations in AY4+5, and to NH24. VG III comprised only one isolate, NXO839, from Gorkha district and was virulent to

Table 1. Isolates of *Xanthomonas oryzae* pv. *oryzae* characterized for their virulence on rice lines containing single resistance genes and gene combinations

Isolate	District	Host	Virulence ^z
NXO741	Dang	Bindeshwori	I
NXO698	Rupendehi	TN-1	I
NXO708	Kapilvastu	Improved	I
NXO697	Rupendehi	Masuli	I
NXO749	Dang	Bindeshwori	I
NXO748	Dang	Bindeshwori	I
NXO742	Dang	Improved	I
NXO691	Kapilvastu	Improved	I
NXO729	Kapilvastu	Radha-17	I
NXO747	Dang	Bindeshwori	I
NXO855	Gorkha	Madesae	I
NXO669	Parsa	Improved	I
NXO853	Lamjung	Mansuli	I
NXO861	Gorkha	Mansuli	I
NXO758	Jhapa	Mansuli	I
NXO849	Gorkha	Sabitri	I
NXO836	Gorkha	Red Anadi	I
NXO842	Tanahu	White Anadi	I
NXO843	Tanahu	White Anadi	I
NXO828	Jhapa	Mansuli	I
NXO810	Sunsari	Bindeshwori	I
NXO827	Jhapa	Dwarf	I
NXO759	Jhapa	Mansuli	I
NXO761	Jhapa	Mansuli	I
NXO763	Jhapa	Mansuli	I
NXO784	Sunsari	Mansuli	I
NXO811	Morang	Mansuli	I
NXO816	Jhapa	B-44	I
NXO817	Jhapa	B-44	I
NXO818	Jhapa	7151	I
NXO823	Morang	Punjabi	I
NXO819	Jhapa	7151	I
NXO850	Gorkha	Mansuli	II
NXO834	Gorkha	Local	II
NXO833	Gorkha	Local	II
NXO820	Jhapa	B-44	II
NXO689	Sunsari	Madrasa	II
NXO730	Kapilvastu	Sabitri	II
NXO694	Rupendehi	Sabitri	II
NXO720	Kapilvastu	Improved	II
NXO750	Dang	Mansuli	II
NXO734	Kapilvastu	Improved	II
NXO839	Gorkha	Red Anadi	III
NXO835	Gorkha	Red Anadi	IV
NXO739	Dang	Radha-9	IV
NXO719	Kapilvastu	Improved	IV
NXO856	Gorkha	Madesae	IV
NXO648	Makwanpur	Local	IV
NXO857	Gorkha	Madesae	IV
NXO789	Sunsari	Radha-12	V

^z Based on information in Figure 2.

Table 2. Resistance gene characteristics of rice lines used to characterize virulence of *Xanthomonas oryzae* pv. *oryzae* isolates in Nepal

Line	Resistance gene(s)
IRBB4	<i>Xa4</i>
IRBB5	<i>xa5</i>
IRBB7	<i>Xa7</i>
IRBB8	<i>xa8</i>
IRBB10	<i>Xa10</i>
IRBB14	<i>Xa14</i>
IRBB21	<i>Xa21</i>
AY4+5	<i>Xa4</i> and <i>xa5</i>
NH21-37-1-1	<i>Xa4</i> , <i>xa5</i> , and <i>xa13</i>
NH24-10-1-3	<i>Xa4</i> , <i>xa5</i> , and <i>Xa21</i>
NH56-1-44-4	<i>Xa4</i> , <i>xa5</i> , <i>xa13</i> , and <i>Xa21</i>
IR24	Susceptible check

the three-gene combination in NH21. VG IV contained six isolates and were avirulent to all four major gene combinations. VG V consisted of one isolate, NXO789, from Sunsari district, and it was virulent on AY4+5, NH21, and NH24, but avirulent on NH56.

The gene combinations in lines AY4+5, NH21, and NH24 were compatible with some isolates and were significantly different ($P < 0.05$). The four-gene combination in NH56 was resistant to all 50 isolates of *X. oryzae* pv. *oryzae* tested (Table 3). Combined analysis of variance indicated significant ($P < 0.05$) interactions between rice lines and isolates. Partitioning of interactions between the two-, three-, and four-gene combinations and the isolates was not significant ($P < 0.05$) (data not shown).

DISCUSSION

Near-isogenic lines having seven different major genes, a two-gene combination, and two three-gene combinations and a four-gene combination for resistance to *X. oryzae* pv. *oryzae*, recently developed by IRRI, were used to analyze virulence of 50 isolates of *X. oryzae* pv. *oryzae* in Nepal. The infection responses induced by the isolates were clear and easily classified into virulent or avirulent on the test rice lines possessing different genes for resistance. All isolates of *X. oryzae* pv. *oryzae* were virulent on the single gene lines, with most isolates producing longer lesion lengths on the near-isogenic lines than the two-, three-, and four-gene combinations. Although resistance gene *Xa21* is reported to be effective and stable against multiple isolates of *X. oryzae* pv. *oryzae* (27), most Nepalese isolates of *X. oryzae* pv. *oryzae*

tested were virulent to the *Xa21* gene in this study. Recent results also indicated that some of *X. oryzae* pv. *oryzae* isolates from Japan and Korea were capable of overcoming the *Xa21* resistance in the donor and engineered lines (16,27; S. H. Choi, *personal communication*). Furthermore, rice lines carrying *Xa21* have not been planted over large areas for a long period. It is also unknown if the resistance conferred by the *Xa21* gene will be durable in a particular location (24).

We identified five virulence groups based on infection responses elicited on rice lines containing combinations of two, three, and four major genes. Pathogenic races of *X. oryzae* pv. *oryzae* are defined by the ability to induce a combination of compatible (susceptible) and incompatible (resistant) reactions when tested on a standard set of differential host cultivars (19,20,21). Even though differences among isolates could be detected, we did not attempt to classify isolates into pathogenic races. In cases where resistance in the host is largely quantitatively inherited, virulence differences among isolates might be of insufficient magnitude to distinguish distinct pathogenic races (8). Indeed, seven near-isogenic lines, IRBB4 (*Xa4*), IRBB5 (*xa5*), IRBB7 (*Xa7*), IRBB8 (*xa8*), IRBB10 (*Xa10*), IRBB14 (*Xa14*), and IRBB21 (*Xa21*), were not effective for characterizing races of *X. oryzae* pv. *oryzae* because they all gave clear compatible reactions to the isolates studied. The two-gene combination AY4+5, three-gene combinations NH21 and NH24, and four-gene combination NH56 were effective for characterizing virulence of *X. oryzae* pv. *oryzae* because they gave clear differential responses. Thus, these gene combination

lines and previously identified local resistant rice cultivars Sabitri and Laxmi (4,5) may be a useful differential set in examining pathogenic races of *X. oryzae* pv. *oryzae* in Nepal.

One of the major goals of rice improvement programs has been to develop rice cultivars with stable resistance to *X. oryzae* pv. *oryzae* (13,17,18). In this study, we

Fig. 1. Percentage of *Xanthomonas oryzae* pv. *oryzae* isolates infecting each rice line when 50 isolates were inoculated to 11 rice lines 40 days after sowing. Virulence was characterized as resistant (<3 cm lesion length) or susceptible (>3 cm lesion length) reaction induced on the rice lines by each isolate 14 days after inoculation. Asterisk indicates significantly different at $P < 0.05$.

Fig. 2. Dendrogram constructed by cluster analysis (UPGMA) using pair-wise comparisons of the 50 isolates of *Xanthomonas oryzae* pv. *oryzae* based on virulence or avirulence patterns produced on the rice lines containing the two-, three-, and four-gene combinations listed in Table 2. Five virulence groups (VG) were identified.

examined the level of resistance to the BB pathogen conferred by single genes individually, a two-gene combination, two three-gene combinations, and a four-gene combination. In our earlier studies (2–5,7), seeds of the lines AY4+5, NH21, NH24, and NH56 were not available. Thus, there was no knowledge at that time concerning relative virulence of *X. oryzae* pv. *oryzae* to these lines containing different gene combinations. Only the lines with two-, three- and four-gene combinations had measurable resistance to *X. oryzae* pv. *oryzae* isolates examined and showed a marked reduction in lesion length. Interestingly, the two-gene combination line, AY4+5, was significantly different from IRBB4 and IRBB5. Furthermore, the two-gene combination of *Xa4* and *xa5* was more resistant than the *Xa4* or *xa5* gene alone. It may be that combining *Xa4* or *xa5* with other resistance genes will also give increased resistance. Although we did not assess *xa13* in a gene line, the NH21 and NH56 lines that had the *xa13* gene were the most resistant and significantly different from NH24, indicating that *xa13* was the main resistance factor operating in these three- and four-gene combination lines.

The information gained in this study has significant implications for regional gene deployment. In fact, none of the single genes used can be expected to control BB in Nepal. The value of combinations with several major genes, on the potential value of *xa13* alone and in combination with other major genes, will prove useful for the long-term goal of pyramiding several resistance genes into well-adapted rice cultivars. Because of the pathogen's ability to rapidly overcome major genes (17,21), one of the most challenging and important areas of research will be to develop a sound strategy for deployment of R-gene-containing lines in order to maximize the

durability of resistance in a particular location. The recent developments in cloning major genes for resistance to bacterial pathogens (25) may enable the incorporation of major genes for resistance to specific pathogen races into genotypes with quantitative resistance (horizontal resistance). The availability of a molecular linkage map of cultivated rice has allowed rapid identification and localization of several markers for BB resistance genes (9,28). By tagging each gene with tightly linked molecular markers (RFLP and RAPD) and conducting extensive genetic analyses, rice breeders and plant pathologists may be able to identify resistant plants derived from crosses with NH56 and local rice germ plasm. Planting mixtures of a combination of three and four major gene combinations, as well as regularly monitoring the pathogen population to assay the occurrence of the pathogenic races that have the ability to overcome one or more deployed resistance genes, also needs to be tested for management of BB in Nepal.

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Table 3. Mean lesion lengths^w caused by five virulence groups^x of *Xanthomonas oryzae* pv. *oryzae* on lines containing single genes and gene combinations in Nepal

Rice line	Resistance gene	Virulence group				
		I	II	III	IV	V
IRBB4	<i>Xa4</i>	13.33 ^y (S) ^z	12.43 (S)	13.00 (S)	11.74 (S)	14.67 (S)
IRBB5	<i>xa5</i>	13.88 (S)	12.92 (S)	15.50 (S)	11.05 (S)	14.33 (S)
IRBB7	<i>Xa7</i>	13.86 (S)	12.55 (S)	14.34 (S)	12.66 (S)	17.17 (S)
IRBB8	<i>xa8</i>	13.52 (S)	11.94 (S)	11.34 (S)	11.81 (S)	16.67 (S)
IRBB10	<i>Xa10</i>	13.79 (S)	13.79 (S)	13.17 (S)	13.13 (S)	13.83 (S)
IRBB14	<i>Xa14</i>	13.84 (S)	13.66 (S)	12.00 (S)	13.96 (S)	14.83 (S)
IRBB21	<i>Xa21</i>	12.70 (S)	10.18 (S)	10.84 (S)	11.98 (S)	13.83 (S)
AY4+5	<i>Xa4</i> and <i>xa5</i>	6.27 (S)	7.48 (S)	2.90 (R)	2.67 (R)	5.83 (S)
NH21-37-1-1	<i>Xa4</i> , <i>xa5</i> , and <i>xa13</i>	1.83 (R)	1.85 (R)	3.14 (S)	1.48 (R)	8.00 (S)
NH24-10-1-3	<i>Xa4</i> , <i>xa5</i> , and <i>Xa21</i>	1.93 (R)	4.07 (S)	1.83 (R)	1.53 (R)	3.33 (S)
NH56-1-44-4	<i>Xa4</i> , <i>xa5</i> , <i>xa13</i> , and <i>Xa21</i>	1.63 (R)	1.35 (R)	1.50 (R)	1.33 (R)	2.17 (R)
IR24	Susceptible check	15.14 (S)	14.93 (S)	13.67 (S)	15.19 (S)	16.67 (S)
LSD _{0.05} = 0.57						
Total isolates		32	10	1	6	1

^w Experimental unit consisted of three plants per bucket. Three fully expanded leaves of each plant per bucket (total nine leaves per experimental unit) were clip-inoculated with each isolate 40 days after sowing. Each treatment was performed twice.

^x Virulence groups were categorized by infection responses on the two-, three-, and four-gene combinations.

^y Overall lesion length means in centimeters (cm) induced by isolate(s) of each virulence group.

^z R = resistant (<3 cm lesion length), and S = susceptible (>3 cm lesion length) were measured 14 days after inoculation.

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